A mixture of TBBPA and TCDD disrupts adipocyte and osteoblast differentiation in human mesenchymal stem cells

Ohta Souichi[1], Kakutani Hideki[2], Yuzuriha Tomohiro[2], Nakao Teruyuki[2]

[1]Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka, Japan, 573-0101, ohta@pharm.setsunan.ac.jp; [2]Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka, Japan, 573-0101,

Introduction

In the worldwide, the obesity population increase rapidly over the past four decades from 1975, and the obesity prevalence reaches 11% in men (266 million) and 15% in women (375 million). If these trends continue, by 2025, global obesity prevalence will reach 18% in men and surpass 21% in women [1]. In Japan, it was reported that the obesity rate was 28.7% in men and 21.3% in women in 2014 [2]. The increment of obesity is caused the rise of outcome risks of various diseases including metabolic syndrome, hyperlipidemia, heart diseases such as coronary artery disease and myocardial infarction, stroke, type 2 diabetes, hypertension, cancers, low grade and chronic inflammation, fatty liver disease, osteoarthritis, respiratory problems and neurodegenerative diseases. So, obesity has been identified as a significant public problem. Obesity is caused by complex interactions among genetic, behavioral and environmental factors, and imbalance between caloric intake and expenditure is considered a key cause of the obesity epidemic. In addition, there is emerging evidence that exposure to environmental endocrine disrupting chemicals (EDCs) may also be an important contributor. These EDCs have been identified as environmental obesogens. Resently, Barker *et al*. proposed the developmental origins of health and disease (DOHaD) hypothesis, which was that various kinds of environmental factor such as nutrition and exposure of chemical substance during pregnancy and postnatal development period have an influence on adolescent and/or adult health and various kinds of disease outcome risks across the lifespan [3]. Moreover, there was high sensibility point against the EDCs, critical point, on embryonic and neonatal period [4]. It is suggested that chemical exposure on embryonic and neonatal period cause obesity and obesity related diseases.

Tetrabromobisphenol A (TBBPA) belongs to brominated flame retardants (BFRs). TBBPA was considered the obesogen through the activation of peroxisome proliferator activated receptor (PPAR) γ on mouse. And, we have previously shown TBBPA facilitated the adipocyte differentiation of 3T3-L1 cells via PPARγ activation. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is famous persistent organic pollutants (POPs) generated from waste incinerator as undesired by-products. TCDD has shown adverse effect for human health, such as induction of xenobiotic enzyme, teratogenicity, carcinogenicity and immunotoxicity. On mice, TCDD interfere with differentiation to osteoblast and osteoclast in bone marrow stem cells, and TCDD inhibited adipogenic differentiation and attenuated the insulin-induced glucose uptake in 3T3-L1 cells. However, the detail effects of TCDD and TBBPA on differentiation balance in human cells differentiated prosperous period still unclear.

Human mesenchymal stem cells (hMSCs) are multipotent cells isolated from bone and adipose tissue. Recently, it was reported that a risk of the osteoporosis rose to decrease the bone density with adipose deposits on bone marrow which was the place where the differentiation of the MSC having the differentiation ability to adipocyte and osteoblast was decided. Thus, there is close to osteoporosis relation to obesity, and it is suggested that to disrupt the differentiation of MSCs involves the induction and exacerbation of the both diseases. In the present study, we investigated the disruption effects of TBBPA and TCDD on the adipocyte or osteoblast differentiation of hMSCs.

Material and methods

1) *Cell cultures*

Human MSC (MSC-R14) was provided by the RIKEN BRC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 3 ng/mL StemBeads FGF2 at 37 ˚C. The cytotoxicity of the chemicals and DMSO was determined using a tetrazolium-based colorimetric assay, the WST-8 kit, according to the manufacturer's

protocol (data not shown).

2) *Adipogenetic induction and oil red O staining*
hMSCs were seeded on a 24-well plate. Two days after confluence (designated as Day 0), cells were treated with adipocyte differentiation medium containing 1 μM dexamethasone, 0.5 mM 3-isobuthyl-1-methylxanthine (IBMX), 200 μM indomethacin and 10 μg/mL insulin. After 3 days (Day 3), the media was replaced and maintained with 10 μg/mL insulin alone to Day 21. The differentiated cells were stained with oil red O to detect lipid droplets in adipocytes. After washing twice with phosphate buffered saline (PBS), cells were fixed with 4% paraformaldehyde at room temperature, and then stained with 3.3 mg/mL oil red O in 60% isopropanol for an hour. Cells were washed with PBS, and observed under an IX71microscope. Stained oil red O was eluted with isopropanol and optical absorbance was measured at a wavelength of 550 nm using a SPECTRA FLUOR for quantitative analyses.

3) *Osteogenetic induction and alizarin red S staining*
At Day 0, cells were treated with osteoblast differentiation medium containing 0.1 μM dexamethasone, 10 mM β-glycerophosphate and 50 μg/mL L-ascorbic acid, and maintained for 21 days. The differentiated cells were assessed by alizarin red S staining for the presence of calcium deposits. Briefly, the cells were fixed with ice cold 70% ethanol, rinsed with distilled water, and then stained with 40 mM alizarin red S dissolved in distilled water (pH 4.2; adjusted with 10% ammonium hydroxide) for 5 min. Cells were washed with distilled water and observed under an IX71microscope. After imaging, the dye was eluted with 10% acetic acid, and the absorbance was measured at 450 nm using a TriStar LB 941 microplate reader.

Result and discussion
To evaluate the effects of TBBPA and TCDD in alone or combination on hMSCs differentiation into adipocyte, we performed using oil red O staining, and quantitated the staining lipid droplet. The number of lipid droplet increased in a dose-dependent manner in the presence of TBBPA. The relative absorbance at 550 nm of 1, 3.3 and 10 μM TBBPA was increased 1.2-, 1.4- and 1.7-fold over the vehicle-treated cells, respectively. On the other hand, the number of lipid droplets decreased in a TCDD higher concentration cells. The relative oil red O absorbance at 550 nm extracted from lipid droplet decreased in a dose-dependent manner in the presence of TCDD: 0.1, 0.3 and 1 nM were 0.82-, 0.73- and 0.67-fold lower compared with the vehicle-treated cells, respectively. In the combination of TBBPA and TBBPA, the nunmber of lipid droplet dose-dependently inhibited by TCDD upregulated by TBBPA in a dose-dependent manner.
To determine whether TBBPA and TCDD induced the expression of adipocyte related genes, adipocyte-specific protein 2 (aP2), and adipocyte related transcription factors, peroxisome proliferator-activated receptor γ (PPARγ), we performed quantitative real-time PCR assay (Fig. 1). TBBPA elevated the mRNA expression of both adipocyte related gene and transcription factors in a dose-dependent manner: aP2 and PPARγat 10 μM TBBPA were 2.9- and 2.6-fold hihger compaerd with the vehicle-treated cells, respectively. TCDD downregulated these mRNA level: aP2 and PPARγ at 1 nM TCDD were 0.19- and 0.38-fold lower compaerd with the vehicle-treated cells, respectively. In the combination assay, these mRNA levels were similar to oil red O staining, especially a significant difference was observd in 0.1 nM TCDD and 1-10 μM TBBPA. The adipocyte differentiated effcet by combination with TBBPA and TCDD became weaker than that of alone. These results indicated that the presence of TBBPA facilitaed the adipocyte differentiation of hMSCs, whereas TCDD suppressed the adipocyte differentiation of hMSCs. And, both compounds having action to disagree with adipocyte differentiation denied each action.
To determine the effects of TBBPA and TCDD on hMSCs differentiation into osteoblast, we first examined alkaline phoapatase (ALP), an early osteoblastic marker, by staining and activity measurement. TBBPA did not influence on osteoblast differentiation On the other hand, the ALP staining and activity of TCDD-induced osteoblast decreased in a dose-dependent manner. The ALP activity at 0.1, 0.3 and 1 nM TCDD was 19.6, 15.6 and 12.1 Units/μg protein, respectively. Moreover, dose-dependent decrement in alizarin red staining was observed: 0.1, 0.3 and 1 nM TCDD were 0.92-, 0.49- and 0.05-fold lower compared with the vehicle-treated cells, respectively. Furthermore, we detected the expression of osteoblast related genes, osteocalcin and osteoblast related transcription factors, runt-related transcription factor 2 (RUNX2) (Fig. 2). These mRNA

levels were decreased in a dose-dependent manner: osteocalcin and RUNX2 at 1 nM TCDD were 0.57- and 0.70-fold lower compaerd with the vehicle-treated cells, respectively. In the combination of TBBPA and TCDD, TBBPA which did not influence osteoblast differentiation suppressed a differentiation induced with TCDD in a dose-dependent manner. These results indicated that TCDD suppressed the osteoblast differentiation of hMSCs. And, TBBPA inhibited the TCDD suppressed osteoblast deffirentiation.

In conclusion, we showed that TBBPA and TCDD were disrupted the differentiation to adipocyte or osteoblkast. The exposure to high level of these chemicals during high-differentiated period, such as fetus and infant, may cause the increment of obesity and obesity related disease outcome risks. Further studies are required to clarify the details of the molecular mechanisms by which pluripotency is disrupted by BFRa and dioxins.

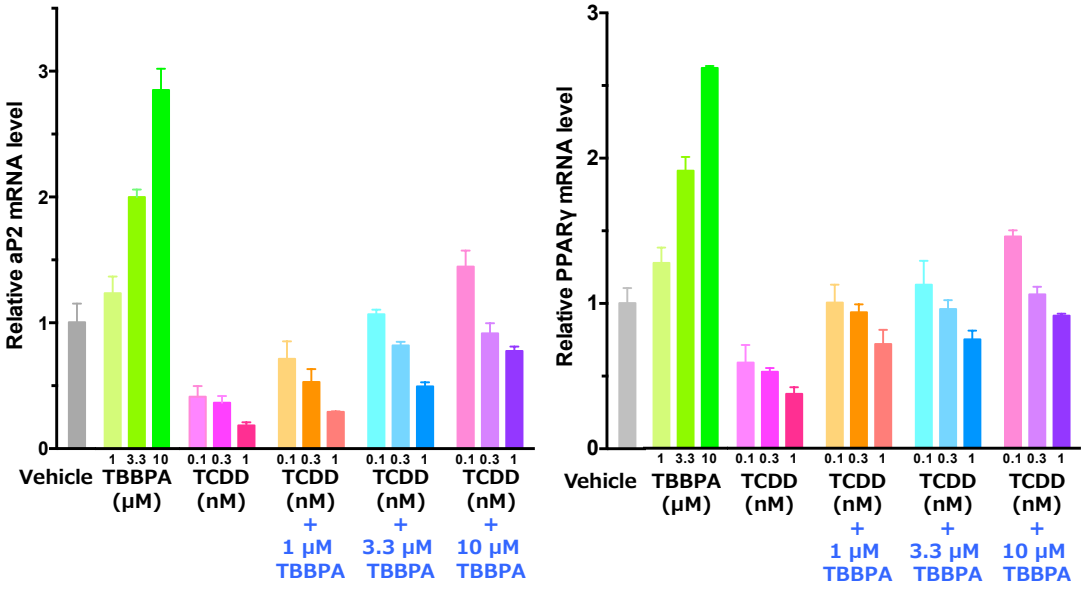

Fig. 1 Effect of TCDD and TBBPA on adipocyte differentiation in hMSCs

hMSCs were differentiated into adipocyte with TBBPA at 1, 3.3 and 10 μM and/or TCDD at 0.1, 0.3, and 1 nM. After 21 days, aP2 (left) and PPARγ (right) mRNA level were measured by realtime-PCR.

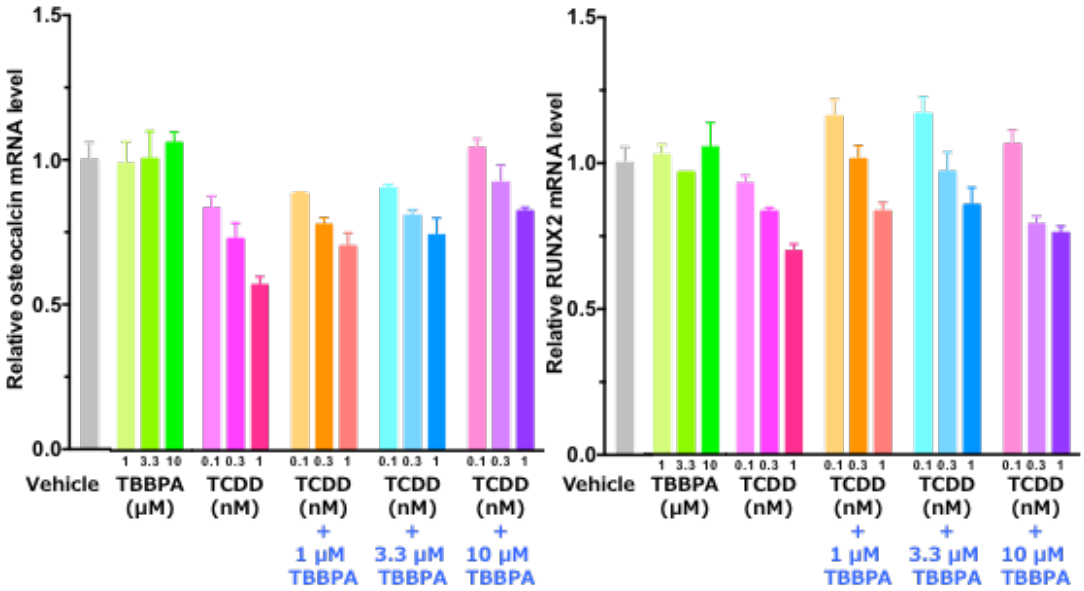

Fig. 2 Effect of TCDD and TBBPA on osteoblast differentiation in hMSCs
hMSCs were differentiated into adipocyte with TBBPA at 1, 3.3 and 10 μM and/or TCDD at 0.1, 0.3, and 1 nM. After 21 days, osteocalcin (left) and RUNX2 (right) mRNA level were measured by realtime-PCR.

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